Exhibit e

Expression of Integrins and Examination of Their Adhesive Function in Normal and Leukemic Hematopoietic Cells

By Jane L. Liesveld, Jill M. Winslow, Karen E. Frediani, Daniel H. Ryan, and Camille N. Abboud

Adhesion of hematopoietic progenitor cells to marrow-derived adherent cells has been noted for erythroid, myeloid, and lymphoid precursors. In this report, we have characterized very late antigen (VLA) integrin expression on normal CD34⁻ marrow progenitors, on leukemic cell lines, and on blasts from patients with acute myelogenous or monocytic leukemias. CD34⁻ progenitor cells expressed the integrin β_1 chain (CD29), VLA-4α (CD49d), and VLA-5α (CD49e). The myeloid lines KG1 and KG1a also expressed CD49d and CD49e as did the Mo7e megakaryoblastic line. CD29, CD18, and CD11a were also present on each of these cell lines. Only the Mo7e line expressed the cytoadhesins GPIIbIIIa or GPIb. Binding of KG1a to marrow stroma was partially inhibited by antibodies to CD49d and its ligand, vascular cell adhesion molecule (VCAM-1). The majority of leukemic blasts studied expressed CD49d and CD49e as

well. Blasts from patients with acute myelomonocytic leukemia consistently bound to stroma at levels greater than 20%, and adhesion to stroma could in some cases be partly inhibited by anti-CD49d. No role for glycosylphosphatidylinositol (GPI)-linked structures was demonstrated in these binding assays because the adhesion of leukemic blasts to stroma was not diminished after treatment with phosphatidylinositol-specific phospholipase C (PI-PLC). These studies indicate that CD34⁻ myeloid progenitors, myeloid leukemic cell lines, and leukemic blasts possess a similar array of VLA integrins. Their functional importance individually or in combination with other mediators of attachment in adhesion, transendothelial migration, and differentiation has yet to be fully elucidated.

© 1993 by The American Society of Hematology.

NORMAL HEMATOPOIESIS in adult humans occurs within the bone marrow (BM) under influences of the marrow microenvironment.[1] These influences, which include cellular,[2] cytokine,[3,4] and extracellular matrix interactions,[5] all contribute to an environment that allows the hematopoietic progenitor cells to proliferate and differentiate normally. In long-term culture of the BM, an in vitro system simulating the in vivo microenvironment, an adherent stromal layer consisting of fibroblasts, macrophages, endothelial cells, and extracellular matrix is important for sustaining hematopoiesis.[6] Early progenitor cells are preferentially associated with the stromal layer,[7,8] and adhesion of early progenitor cells to marrow-derived adherent cells has been previously documented for erythroid,[9] myeloid,[10,11] and lymphoid[12,13] precursors. Cell-cell and cell-matrix interactions between the BM microenvironment and hematopoietic progenitors likely play an important role in the proliferation and maturation required for normal hematopoiesis.

A number of potential cell adhesion molecules, which may mediate essential cell-cell or cell-matrix interactions, have been identified in experimental models of BM.[14-18] The integrin superfamily of cell adhesion molecules plays a key role

in development, immune response, platelet aggregation, and tissue repair.[19-21] and it is currently the focus of intense study regarding its potential role in myeloid[17] and lymphoid hematopoiesis.[12-15] Integrins are heterodimer glycoproteins consisting of noncovalently linked α and β chains. They are classified according to their β chain into the very late antigen (VLA) integrins (β_1), leukocyte integrins (β_2), cytoadhesin integrins (β_3), and additional molecules expressing β_4, β_5, β_6, β_7 (β_p), or β_8 chains. The β_1(VLA) subfamily consists primarily of receptors for cell matrix components and is expressed in both hematopoietic and nonhematopoietic cells.[20-22] The seven members of the VLA subfamily are distinguished by the association of different α-chains with the common integrin β_1-chain.[19,21-23] Hematopoietic expression of the VLA integrins includes VLA-1, VLA-2, and VLA-6 (CD49a, b, e) presence on T cells,[23-25] and VLA-2 and VLA-6 expression on platelets.[?-?] VLA-4 (CD49d) and VLA-5 (CD49e) are more widely expressed within the hematopoietic system.[12,14,16,17,25]

We have previously investigated the interaction of myeloid progenitors with BM stroma in vitro and reported that a subset of human marrow cells that possesses the CD34 antigen binds to marrow stromal monolayers[10] and, to a lesser extent, to extracellular matrices of marrow and to fibronectin and laminin.[11] We also reported that both normal CD34⁺ myeloid progenitors and myeloblastic cell lines (KG1 and KG1a) express the β_2 integrin (CD18), but the adhesive interactions between these myeloid progenitors and marrow stromal layers were not inhibited by anti-CD18 monoclonal antibodies (MoAbs) with functional blocking properties.[11] In this report, we examine further the expression of integrins on normal CD34⁻ myeloid progenitors as well as on the leukemic cell lines KG1, KG1a, and Mo7e and myeloblasts obtained from leukemic patients. We also investigate the possible involvement of the VLA integrins in adhesive interactions between myeloid precursors and the marrow microenvironment through studies of binding inhibition.

MATERIALS AND METHODS

Separation and Culture

Marrow CD34+ myeloid precursors. A sterile two-step flow cytometric technique used to isolate CD34+ marrow cells has been described previously.[10] BM aspirates were obtained from normal volunteer donors in accordance with institutional guidelines of the Research Subjects Review Board of the University of Rochester. 5×10^7-light-density marrow cells per sample were stained with a 1:20 dilution of fluorescein isothiocyanate (FITC)–conjugated HPCA-1, an antibody that recognizes the CD34 antigen (Becton Dickinson, Mountain View, CA), and with phycoerythrin (PE)–conjugated anti-CD10 (CALLA antigen: Coulter, Hialeah, FL). One to 5% of cells positive for CD34 and negative for CD10. Cells were then sorted aseptically using an EPICS C flow cytometer (Coulter). An initial sort performed at high speed resulted in a population that was 60% to 70% CD34+. A second slow-speed sort resulted in a 96% to 99% pure CD34+CD10− population. These cells, which were 95% viable at the conclusion of the second sort, constituted the purified CD34+ myeloid precursors subsequently used in binding assays and indirect immunofluorescence phenotyping studies. At the completion of the sort, centrifugation was performed to remove sorting sheath fluid, and the cells were resuspended in RPMI medium with 20% fetal bovine serum (FBS) for overnight storage at 37°C if further studies could not be conducted soon after sorting conclusion.

In certain experiments. CD34+ cells were isolated by an avidin-biotin column adsorption technique (CellPro, Inc. Bothell, WA).[26]

Cell lines. The leukemic cell lines KG1. KG1a. and Mo7e were obtained from Dr D.W. Golde (Memorial Sloan Kettering, Rye, NY). KG1 and KG1a were maintained in RPMI-1640 (GIBCO, Grand Island, NY) culture medium with 10% FBS (Hyclone, Logan, UT). Mo7e is an interleukin-3 (IL-3)–dependent cell line and was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO) with 20% FBS and 50 pmol/L IL-3 (kindly provided by Dr S. Clark, Genetics Institute, Cambridge, MA).

Leukemic patient samples. Marrow or blood obtained with informed consent was layered over Ficoll-Hypaque (specific gravity 1.77: Pharmacia, Piscataway, NJ). Light-density interface cells were washed twice and resuspended in RPMI with 10% FBS. Samples were used in binding assays only if they had greater than 80% blasts.

Stromal cell layers. Marrow stromal cell layers were established from BM aspirates from normal volunteers and were cultured and passaged as previously described.[10] Briefly, light-density BM cells were cultured in McCoy's 5A culture medium (GIBCO) supplemented with 12.5% FBS. 12.5% horse serum (Hyclone), and 1 μmol/L hydrocortisone (Sigma Chemical Co. St. Louis. MO). These adherent cell layers were used in binding assays only after passage of more than three times. At final passage, the adherent layers were seeded into either Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ) for cell line chromium-binding studies or into 35-mm tissue culture plates (Corning, Corning, NY) for CD34+ progenitor cell adhesion assays. The adherent layers were allowed 5 to 7 days of incubation after the final passage to ensure adequate confluent matrix formation for cell-binding assays.

Antibodies and Reagents

Antibodies and additional reagents were obtained from Becton Dickinson Immunocytometry Systems, Mountain View, CA: (HPCA-1 [CD34. IgG1] and FITC-conjugated goat antimouse isotype control for IgG1). Coulter Immunology, Hialeah, FL: (CD29 [VLA β_1 chain, IgG1], CD10PE, and goat antimouse isotype controls for IgG1, IgG2a, and IgG3). Fisher Scientific, Orangeburg, NJ: (FITC-conjugated isotype specific goat antimouse IgG1, PE-conjugated isotype specific goat antimouse IgG2b and IgG3). Telios Pharmaceuticals, San Diego,

CA: (CD49b [VLA-2 α chain. IgG1]. CD49c [VLA-3 α chain. IgG1], and CD49e [VLA-5 α chain. IgG3]). AMAC. Westbrook. ME: (CD49d [VLA-4 α chain. IgG1]. CD49f [VLA-6 α chain. IgG2a]. CD61 [GPIIIa or β_3. IgG1]. and CD34 FITC conjugate). Tago. Burlingame. CA: (FITC-conjugated F(ab')2 goat anti-mouse Ig. and PE-conjugated F(ab')2 goat anti-mouse Ig). Antibodies L1 (anti-LFA-1 α chain. CD11a). 44 alpha (anti-Moα-chain. CD11b). L29 (anti-p150.95 α-chain. CD11c). and 10F12 (anti-β_2-chain. CD18) were gifts of Dr A. Arnaout (Massachusetts General Hospital, Boston, MA). Antibody 12.8. an IgM molecule recognizing CD34. was a gift of Dr R. Berenson. Seattle. WA. Anti-VCAM (4B9. IgG1) was a gift from Dr J. Harlan (Harborview Medical Center. Seattle. WA). For diagnostic immunophenotyping of leukemic blasts. anti-CD14. anti-CD19. anti-CD33. and anti-CD34 were obtained from Coulter. Antibodies AP1 (anti-GPIb), AP2 (anti-GPIIbIIIa). AP4 (anti-GPIIb). and AP5 (anti-GPIIa) were gifts of Dr T.J. Kunicki (The Blood Center of Southeastern Wisconsin. Milwaukee). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from ICN Biomedicals (Costa Mesa. CA). Granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-3 were gifts of Dr S. Clark.

Adhesion Protein Expression

Cell lines were stained for indirect immunofluorescence by incubating with antibody to the adhesion protein at 4°C for 30 minutes followed by three washes in PBS. Cells were then incubated at 4°C for 30 minutes with FITC-conjugated goat anti-mouse Ig. After the final wash. cells were resuspended in 1% paraformaldehyde in PBS at 4°C for flow cytometry analysis. Stained cells were analyzed on an EPICS C flow cytometer. The mean log green fluorescence channel of the cell lines was determined directly from a single parameter histogram. The mean log fluorescence channel was converted to linear equivalents as previously described.[27] In order to estimate the fluorescence intensity specifically due to the test adhesion protein antibody. the mean fluorescence intensity of cells stained with isotype-specific control antibody was subtracted from that of cells stained with the adhesion protein antibody to obtain the final mean fluorescence intensity measurement. To ensure that only specific receptor expression was evaluated. only fluorescence measurements ≥ 5 were defined as positive after subtraction of background staining.

For phenotyping of adhesion proteins on normal CD34+ myeloid progenitors. two-color immunofluorescence was used with light-density marrow cells stained with an adhesion marker versus CD34. 2×10^6-light-density bone marrow cells were incubated with 12.8 anti-CD34 and unconjugated test antibody for 30 minutes at 4°C in PBS plus 20% human AB serum and then washed twice with cold PBS. The cells were then incubated with FITC-conjugated goat anti-mouse IgG specific for the isotype of the unconjugated "test" antibody used and with goat antimouse IgM PE. After staining, cells were resuspended in 1% paraformaldehyde in PBS and kept at 4°C for flow cytometry analysis. Stained cells were analyzed on an EPICS Profile flow cytometer (Coulter) using standard light scatter gates for mononuclear cells to obtain a two-parameter histogram of log green fluorescence (FITC) versus log red fluorescence (PE).

Binding Assays

Chromium binding assay. Binding experiments with cell lines were performed as previously described[22] with minor modifications. KG1. KG1a. or Mo7e cells (5×10^6) were labeled with 100 μCi ^{51}Cr (Amersham. Arlington Heights, IL) for 30 minutes at room temperature. Cells were then washed three times in more than 10 vol of PBS. Cells were then resuspended in RPMI-1640 medium with 10% FBS at a concentration of 1.0×10^6 cells/mL. One-half milliliter (5 $\times 10^5$ cells) were plated over the stromal layer or plastic-coated 24-well tissue culture plate for 2 hours at 37°C. The medium and non-

with the degree of binding to stroma nor did possession of CD14. a monocytic marker. correlate with degree of binding to tissue culture plastic. In five cases. the effect of PI-PLC on leukemic blast binding to stroma was investigated. Cells were treated with 120 mU PI-PLC for 30 minutes at 37°C. washed once. and plated in the previously described adherence assay. As shown in Fig 3. in only two cases did mean binding decrease in presence of PI-PLC: by 21% in sample 3 and by 27% in sample 5.

Inhibition of Adhesion by Blocking Antibodies

To study which of the expressed adhesion proteins might be involved with adhesion of myeloid progenitors to the BM stroma. we used an in vitro model of stromal adhesion to conduct inhibition studies with blocking antibodies to the integrins of interest.

Each of the adhesion receptor antibodies used in the inhibition experiments blocks adhesion at the concentration used. CD29 and CD49d antibodies inhibited adhesion of a B-cell line. NALM-6. to BM fibroblasts by 57% and 52%. respectively.[12] The CD49e and anti-VCAM antibodies were used at a concentration previously demonstrated to show blocking of VLA-5–dependent and VCAM-dependent adhesion. respectively.

CD34+ cell lines and leukemic myeloblasts. The effect of various blocking antibodies on adhesion of the myeloblastic cell lines KG1. KG1a. and Mo7e to bone marrow stroma is shown in Fig 4. Binding of the KG1a. KG1. and Mo7e cells to normal bone marrow stroma was not significantly decreased by anti-CD49e (anti-VLA-5α) or CD29 (anti-β_1). Anti-CD49d (anti-VLA-4α). and anti-VCAM had no effect on the adhesion of KG1 or Mo7e to marrow stromal layers. As shown in the case of Mo7e. the combination of anti-VLA4-α and anti-CD29 did not block adhesion either. In contrast. KG1a binding to marrow stroma was partially inhibited by both anti-VLA-4α and anti-VCAM (*P* < .05 by paired t-test) (Fig 4A). The combination of these two anti-

bodies did not result in greater inhibition than either used alone. No difference in the degree of inhibition was observed between addition of the test antibody at the time of target cell incubation over stromal layers versus preincubation of antibody with ligand-containing cells for 30 minutes at 4°C.

In three cases of myeloid leukemia where sufficient blasts were obtained to study effects of antibodies to VLA integrins on stromal adhesion. anti-VLA-4α inhibited blast binding by a mean of 18% ± 4.9% (range 10% to 27%). In two cases studied. anti-VLA-5α did not result in significant inhibition of blast binding.

Normal CD34+ progenitors. We have previously shown that normal CD34+ progenitors attach to stromal layers using a binding assay in which the readout is numbers of CFU-GM colonies.[10] Using this same assay. inhibition of adhesion of normal CD34+ progenitors to BM stroma was examined. When the binding assay was performed in the presence of anti-CD29 (β_1) (1:100 dilution of 4B4 antibody). inhibition of adhesion of normal progenitors to marrow stroma was 53% (±4.2%. n = 3). Data from these three experiments are shown in Fig 5A. Addition of anti-VLA-4α chain antibody did not enhance the inhibition seen with anti-β_1 alone.

In addition. to assess effects of these blocking antibodies upon the entire population of CD34+ cells. [51]Cr labeling of column-adsorbed CD34+ cells was performed followed by the standard adhesion assay. As shown in Fig 5B. anti-CD29 (VLA-β_1 chain) gave significant binding inhibition in this population as well (*P* < .01 by paired t-testing). whereas anti-VLA-4α. anti-VCAM. and anti-VLA-5α did not.

Effects of cytokines on binding of leukemic cell lines. To assess whether incubation of progenitor or leukemic cells with growth factors would enhance adhesion as has been previously reported for transplanted marrow in murine models.[28] or to examine whether exposure of the stromal layers to inflammatory/immune modulators would influence adhesion. adhesion assays were performed after incubation with appropriate cytokines. As shown in Table 4. neither IL-3 nor

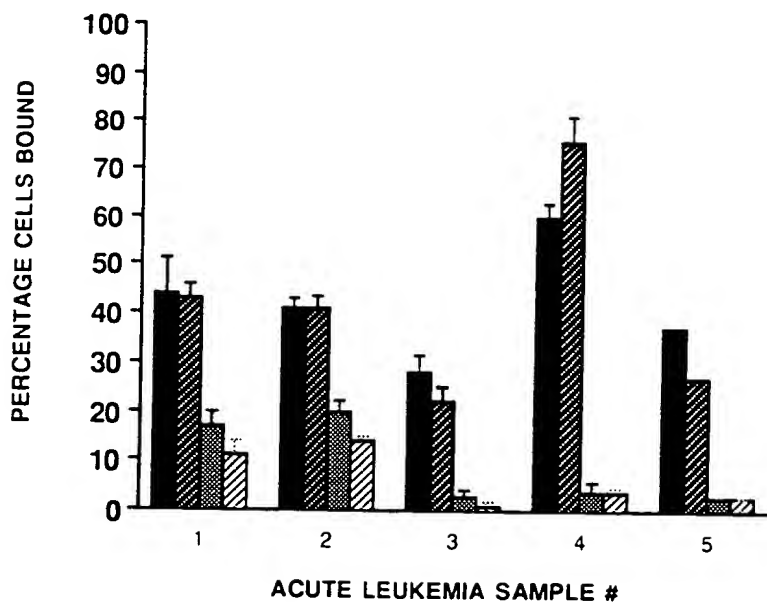Fig 3. Shown is the percentage of myeloblasts. bound to stromal or plastic layers in the presence or absence of PI-PLC treatment in five cases of acute myeloblastic leukemia (AML). Similar treatment of blood monocytes with PI-PLC decreased CD14 antigen density by greater than 50%. N = 3 except for sample No. 5 performed in duplicate. (■), Stroma − PLC; (▨), stroma + PLC; (▩), plastic − PLC; (▨), plastic + PLC.
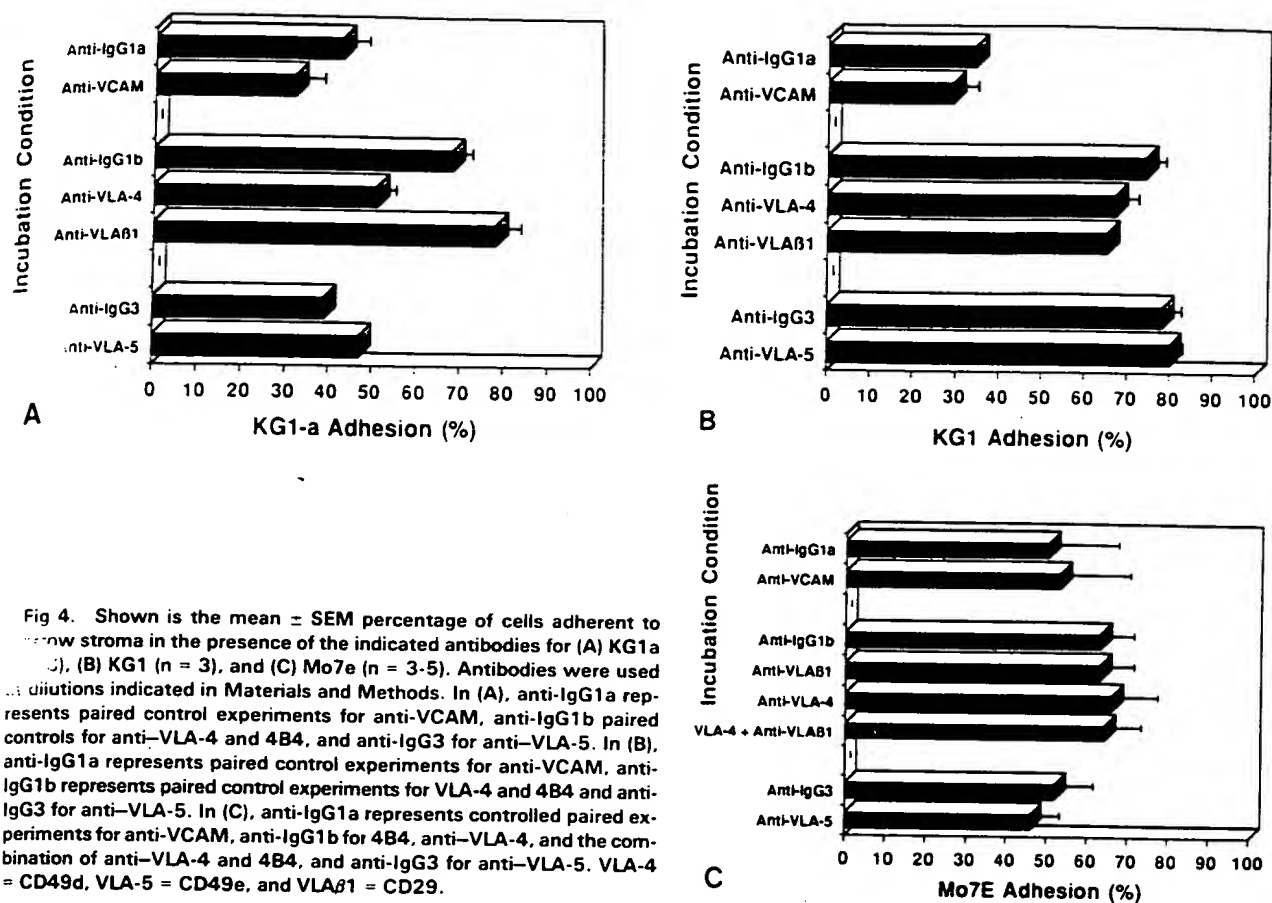
Fig 4. Shown is the mean ± SEM percentage of cells adherent to ... ow stroma in the presence of the indicated antibodies for (A) KG1a ...), (B) KG1 (n = 3), and (C) Mo7e (n = 3-5). Antibodies were used ... dilutions indicated in Materials and Methods. In (A), anti-IgG1a represents paired control experiments for anti-VCAM, anti-IgG1b paired controls for anti–VLA-4 and 4B4, and anti-IgG3 for anti–VLA-5. In (B), anti-IgG1a represents paired control experiments for anti-VCAM, anti-IgG1b represents paired control experiments for VLA-4 and 4B4 and anti-IgG3 for anti–VLA-5. In (C), anti-IgG1a represents controlled paired experiments for anti-VCAM, anti-IgG1b for 4B4, anti–VLA-4, and the combination of anti–VLA-4 and 4B4, and anti-IgG3 for anti–VLA-5. VLA-4 = CD49d, VLA-5 = CD49e, and VLAβ1 = CD29.

... !-CSF at concentrations of 50 U/mL and 100 U/mL, re-... ctively, affected binding of KG1a or KG1 to marrow stromal layers. Also, pretreatment of stromal layers with 10^{-8} mol/L PMA (phorbol myristate acetate) or 10 U/mL IL-1α did not alter the degree of adhesion of the KG1a or Mo7e cell lines nor of normal CD34$^+$ myeloid progenitors to such layers (Table 5).

DISCUSSION

The β_1 (VLA) integrins are generally associated with adhesion to extracellular matrix components. In this study, it has ... n documented that early myeloid cells, including normal ... D34$^+$ progenitors, early leukemic cell lines, and acute leukemia myeloblasts all possess a similar array of VLA integrins. Most prominently expressed are CD29 (the common β_1) chain; CD49e (VLA-5α), a receptor for the RGDS cell-binding site of fibronectin; and CD49d (VLA-4α), which can bind to fibronectin domains[29,30] as well as to a cell-associated ligand, VCAM-1.[31] CD49b and CD49c were minimally expressed on all of these cell types. VLA-6, a receptor for laminin, was present only on established leukemic cell lines, and ... ost prominently on Mo7e, a line with some megakaryo-... stic properties. We have shown previously that normal ... D34$^+$ myeloid progenitors possess CD18,[11] and here it is shown that immature cell lines (CD34$^+$) also possess CD18 and CD11a. No significant expression of cytoadhesins was found on the three cell lines tested except on Mo7e, which

has megakaryocytic properties. Normal CD34$^+$ progenitors did not possess the β_3 chain (CD61, vitronectin β receptor/gpIIIa).[32]

The spectrum of β-integrin antigen expression documented here is in keeping with that noted by others using different assay methods. Saeland et al[33] have reported a similar array of integrin antigen expression on CD34$^+$ cells from marrow and cord blood, and a repertoire of integrin expression during erythroid maturation has also been described.[34] Soligo et al.[17] using an immunohistochemical detection method, also found VLA-4 presence on immature hematopoietic cells and restriction of β_3 cytoadhesin molecules to megakaryocytes and platelets when unfractionated BM was studied. They also noted only a minor role for β_2 integrins in early hematopoiesis and found variability of β_3 integrin expression on acute myelogenous leukemia specimens, whereas VLA-2, VLA-3, and VLA-6 were absent on nonlymphoid progenitors.[17]

In the study presented here, VLA-5α (CD49e) was seen on all early myeloid cell types (normal CD34$^+$ progenitors, leukemic cell lines, and acute leukemia myeloblasts) with a relative increase seen in some cases of acute myelocytic leukemia (AML), probably reflecting the cell-maturation spectrum involved in leukemogenesis.[17] In addition to VLA-5α and the common β_1 chain (CD29), only VLA-4α (CD49d) was prominently expressed on normal early myeloid cells. Within the acute leukemia samples studied, VLA-4α expression did not seem to correlate with maturation as even an
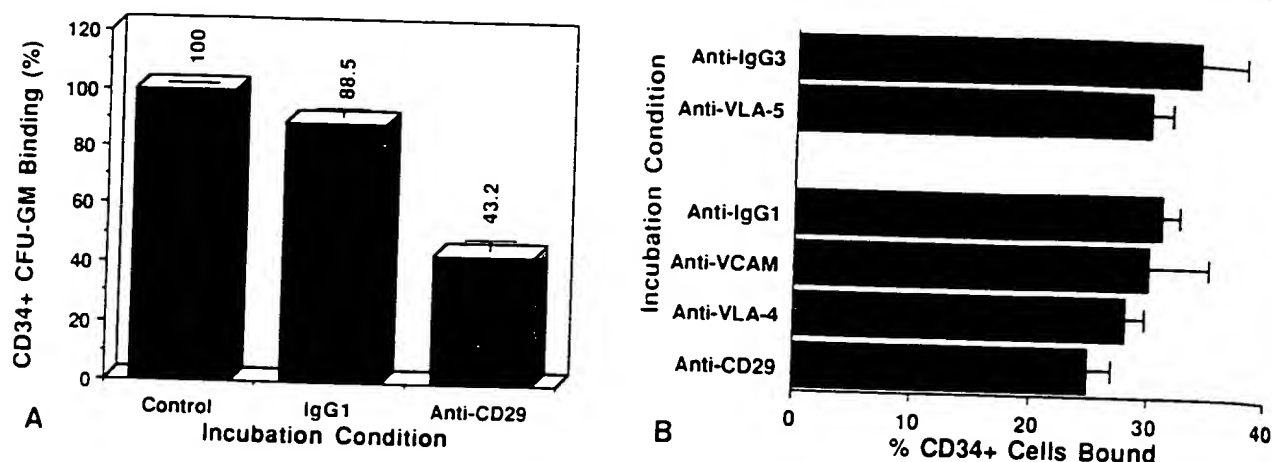
Fig 5. (A) Shown are the mean ± SEM percentage of CFU-GM bound to marrow stroma in the presence of anti-CD29 (anti-β_1) or in the presence of an IgG1 control antibody as compared to binding to stroma without antibody present (100%); n = 3 for all conditions. (B) The percentage of CD34⁻ cells bound to marrow stroma in the presence of the indicated antibodies is shown with all data obtained in a parallel fashion with the appropriate isotype specific control antibody; n = 4 for all IgG1-paired conditions and n = 3 for VLA-5/IgG3. VLA-4 = CD49d and VLA-5 = CD49e.

M3 specimen with no CD34⁺ blasts expressed significant levels of VLA-4α. VLA-4 has been found on cultured T lymphocytes to function as a receptor for the heparin-II and IIICS domains of fibronectin.[29] VLA-4α has also been found to be a ligand for VCAM-1. an adhesion molecule of the immunoglobulin gene superfamily found on endothelial cells stimulated with IL-1α. lipopolysaccharide. or tumor necrosis factor α. It is also constitutively expressed on dendritic cells. renal tubular epithelial cells. and tissue macrophages.[31] VCAM has also been found on marrow stromal cell layers such as those used here.[35]

To what extent CD49d (VLA-4α) or CD49e (VLA-5α) antigens participate in homing phenomena or in attachment of myeloid progenitors to marrow microenvironmental components remains unknown. The data presented here show no role for VLA-5α in adhesion of CD34⁺ cells to stroma as measured in a specific adhesion assay. This is in keeping with previous studies that show no role for RGD sequences in such adhesion processes.[36,37] In contrast. RGD sequences have

been found to have a role in adhesion of erythroid progenitors to marrow.[33]

Antibody inhibition data shown here suggest a role for CD29 (β_1 integrin) in adherence of CD34⁻ CFU-GM–forming cells to marrow stroma and participation of VLA-4α (CD49d) in adhesion of some myeloid progenitors (KG1a cell line and three samples from leukemia patients) to stroma. Data shown here with the KG1a cell line would suggest that VCAM-1 on -human marrow stromal cells may participate in leukemic blast adhesion as the ligand for VLA-4. Such an interaction has also been noted for binding of lymphocytes or lymphocyte progenitors to endothelial cells.[38,39] to murine marrow stromal cells.[13,40] or to human marrow fibroblasts expressing V-CAM-1.[12] and. more recently. for adhesion of myeloid and erythroid progenitors to marrow stromal cells.[35]

In no instance did antibodies to VCAM-1. VLA-4α (CD49d). β_1 (CD29). or other VLA integrins completely inhibit myeloid progenitor adhesion to marrow stroma. and, in most cases. inhibition of adhesion noted was minimal. This might suggest a role for other classes of integrins or other types of adhesion receptors in the interaction of leu-

Table 4. Effect of GM-CSF and IL-3 on GK1a and KG1 Adhesion

	KG1a	KG1a – GM-CSF	KG1a	KG1a + IL-3
Stroma	65 ± 3.0	63 ± 6.1	63 ± 11	68 ± 9.0
Plastic	1.3 ± 0.6	2.0 ± 1.0	3.8 ± 1.2	4.0 ± 0.7

	KG1	KG1 – GM-CSF	KG1	KG1 + IL-3
Stroma	68 ± 2.9	74 ± 3.5	18 ± 5	19 ± 6
Plastic	2.7 ± 0.6	3.7 ± 1.2	ND	ND

Data represent the mean ± standard deviation percentage of the indicated cell line which adhered to marrow stromal layers or plastic in the presence or absence of GM-CSF (100 U/mL) or IL-3 (50 U/mL). Experiments were performed in a paired fashion with n = 3 for all conditions except for KG1a + IL-3 experiments and their controls for which n = 5.

Abbreviation: ND = not done.

Table 5. Effect of IL-1α and Phorbol Myristate Acetate (PMA) on Cell Adhesion

	CD34⁺ Progenitors	KG1a	Mo7e
Stroma	14 ± 3.2 (n = 3)	23 ± 9.5 (n = 4)	43 ± 9 (n = 3)
Stroma + IL-1α	17 ± 3.5 (n = 3)	26 ± 8.6 (n = 4)	49 ± 12 (n = 3)
Stroma	38 ± .89 (n = 3)	41 ± 4.7 (n = 3)	48 (n = 2)
Stroma + PMA	41 ± 2.3 (n = 3)	42 ± 4.7 (n = 3)	53 (n = 2)

Data shown are mean ± SEM percentage of the indicated cell type bound to stromal layers grown in the presence or absence of 10 U/mL IL-1α or 10^{-8} mol/L PMA for 24 hours. Experiments were performed in a paired fashion. n = number of experiments.

kemia cells with marrow. These might include heparin-binding receptors. proteoglycans. L-selectin and its mucin-like ligand.[33,41] CD44-hyaluronate receptors.[42] hemonectin.[43] thrombospondin.[44] other collagen receptors.[37] or other fibronectin receptors.[45] A role for the 33/66-Kd carboxy-terminal heparin-binding cell adhesion domains of fibronectin in interactions between primitive marrow progenitors and intact irradiated marrow stroma has been found.[46] This interaction appeared to involve the FN-C/H II site. Also. other anchored

ıh factors such as membrane-bound basic fibroblast ıh factor.[47] macrophage colony-stimulating factor (M-CSF. CSF-1).[48] or c-kit ligand (stem cell factor)[49] may participate in this adhesion process. Figure 6 illustrates schematically some of the possible receptor-ligand interactions that might contribute to precursor (CD34$^+$) attachment to marrow stroma.
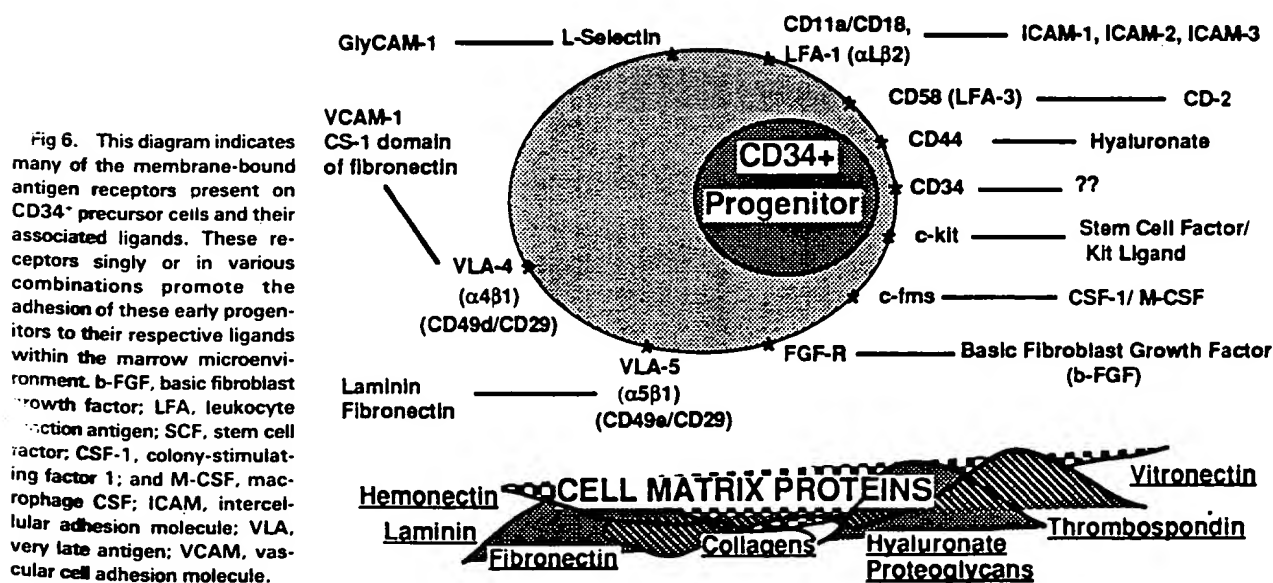
The lack of inhibition seen with anti-CD29 in the case of myeloid/megakaryoblastic cell lines does not necessarily rule out a role for β_1 integrins in their adhesion to stroma. Changes in conformation of some integrin receptors after antibody

ıng can "activate" or alter the affinity of ligand recognition. This has been reported for another monoclonal antibody to CD29 (8A2) that stimulated the binding of U937 cells to CHO cells transfected with VCAM-1 complementary DNA (cDNA)[50] and for glycoprotein IIbIIIa.$\alpha_{IIb}\beta_3$.[51] Whether such a phenomenon might be operational in the adhesion of myeloid leukemic progenitors to stroma can only be speculated on. It is also possible that to detect the role of the CD49d(VLA-4α)/VCAM complex in mediating progenitor adhesion to the microenvironment. one may have to maximize the stromal expression of VCAM-1 by cytokine combinations. as recently shown by Simmons et al.[35]

in this work. the presence of a similar array of VLA integrins on freshly isolated leukemia blasts as on tissue culture-adapted cell lines and normal CD34$^+$ progenitors was identified. Leukemic marrow blasts demonstrated. on average, a comparable degree of binding to marrow stromal layers as did these other cell types. Although the number of samples reported upon here is small. the presence of CD49d and CD49e was consistently noted. and the degree of blast adhesion to stromal layers was independent of subtype: ie. myelogenous versus monocytic versus promyelocytic. In those cases where adhesion inhibition was studied with antibody to CD49d. a small degree of inhibition was noted.

This ability of leukemic blasts to adhere to marrow stromal layers is surprising given the common presence of blood blasts in these cases even when marrow cellularity does not suggest a crowding effect. Because VLA integrin expression appears similar to that of normal progenitors. it is possible that blast egress occurs because of altered function or affinity of these receptors for their respective ligands. It is also possible that these blasts lack adhesive receptors of other uncharacterized classes as has been described for progenitors in chronic myelogenous leukemia (CML).[52] Unlike the situation in CML. the acute myelo(monocytic) blast adhesion to stroma was not consistently PI-PLC linked.[53] Another explanation for blast exit from the marrow would be altered receptor affinity or changes in integrin phenotype on egress. Such possibilities could be explored by comparing the adhesive properties of circulating blood blasts to marrow blasts. Hematopoietic growth factors such as GM-CSF and IL-3 have been reported to influence adhesion or aggregation of mature granulocytes and monocytes.[54,55] but their presence did not influence binding of early myeloid cell lines to marrow stromal layers in our hands.

As cells of the granulocyte lineage mature. they lose VLA-4α. whereas monocytes and eosinophils[56] retain this protein. Changes in integrin phenotype with blast differentiation may partially explain the propensity of monoblasts for tissue invasion. Other factors regulating acute myelogenous leukemia blast traffic may relate to their responses to chemotactic factors or their ability to elaborate collagenases[46] or enzymes

Fig 6. This diagram indicates many of the membrane-bound antigen receptors present on CD34$^+$ precursor cells and their associated ligands. These receptors singly or in various combinations promote the adhesion of these early progenitors to their respective ligands within the marrow microenvironment. b-FGF, basic fibroblast growth factor; LFA, leukocyte adhesion antigen; SCF, stem cell factor; CSF-1, colony-stimulating factor 1; and M-CSF, macrophage CSF; ICAM, intercellular adhesion molecule; VLA, very late antigen; VCAM, vascular cell adhesion molecule.

GlyCAM-1 ——— L-Selectin
CD11a/CD18, ——— ICAM-1, ICAM-2, ICAM-3
LFA-1 (αLβ2)
CD58 (LFA-3) ——— CD-2
VCAM-1 CS-1 domain of fibronectin
CD44 ——— Hyaluronate
CD34 ——— ??
c-kit ——— Stem Cell Factor/ Kit Ligand
VLA-4 (α4β1) (CD49d/CD29)
c-fms ——— CSF-1/ M-CSF
FGF-R ——— Basic Fibroblast Growth Factor (b-FGF)
VLA-5 (α5β1) (CD49e/CD29)
Laminin Fibronectin

CELL MATRIX PROTEINS
Hemonectin Laminin Fibronectin Collagens Hyaluronate Proteoglycans
Vitronectin Thrombospondin

CD34+ Progenitor

such as the human heparin-binding elastase homologue (CAP37. azurocidin) that mediates reversible fibroblast and endothelial cell contractility.[57] thereby facilitating cell egress into extravascular spaces. Further studies will be required to assess the role of the VLA integrins in leukemia blast egress from the marrow and any role that they might play in stem cell homing during transplantation.[58]

REFERENCES

1. Lichtman MA: The ultrastructure of the hemopoietic environment of the marrow: A review. Exp Hematol 9:391. 1981

2. Torok-Storb B: Cellular interactions. Blood 73:373. 1988

3. Golde DW. Gasson JC: Hormones that stimulate the growth of blood cells. Sci Am 259:62. 1988

4. Nathan C, Sporn M: Cytokines in context. J Cell Biol 113:981. 1991

5. Gordon MY: Extracellular matrix of the marrow microenvironment. Br J Haematol 70:1. 1988

6. Dexter TM. Allen TC. Lajtha LG: Conditions controlling the proliferation of hematopoietic stem cells in vitro. J Cell Physiol 91:335. 1977

7. Coulombel L. Eaves AC. Eaves CJ: Enzymatic treatment of long-term marrow cultures reveals the preferential location of primitive hematopoietic progenitors in the adherent layer. Blood 62:291. 1983

8. Slovick FT. Abboud CN. Brennan JK. Lichtman MA: Survival of granulocytic progenitors in the non-adherent and adherent compartments of human long-term marrow cultures. Exp Hematol 12:327. 1984

9. Tsai S. Sieff CA. Nathan DG: Stromal cell-associated erythropoiesis. Blood 67:1418. 1986

10. Liesveld JL, Abboud CN. Duerst RE. Ryan DH. Brennan JK. Lichtman MA: Characterization of human marrow stromal cells: Role in progenitor cell binding and granulopoiesis. Blood 73:1794. 1989

11. Liesveld JL, Winslow JM. Kempski MC. Ryan DH. Brennan JK. Abboud CN: Adhesive interactions of normal and leukemic human CD34+ myeloid progenitors: Role of marrow stromal. fibroblast. and cytomatrix components. Exp Hematol 19:63. 1991

12. Ryan DH. Nuccie BL. Abboud CN. Winslow JM: VLA-4 and VCAM-1 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. J Clin Invest 88:995, 1991

13. Miyake K. Weissman IL. Greenberger JS. Kincade PW: Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J Exp Med 173:599. 1991

14. Guan JL, Hynes RO: Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell 60:53. 1990

15. Kansas GS. Muirhead MJ. Dailey MO: Expression of the CD11/CD18. leukocyte adhesion molecule 1. and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. Blood 76:2483. 1990

16. Rosemblatt M. Vuillet-Gaugier MH. Leroy C. Coulombel L: Coexpression of two fibronectin receptors. VLA-4 and VLA-5 by immature human erythroblastic precursor cells. J Clin Invest 87:6. 1991

17. Soligo D. Schiro R. Luksch R. Manara G. Quirici N. Parravicine C. Deliliers GL: Expression of integrins in human bone marrow. Br J Haematol 76:323. 1990

18. Witte PL. Robinson M. Henley A. Low MG. Stiers DL. Perkins S. Fleischman RA. Kincade PW: Relationships between B-lineage lymphocytes and stromal cells in long-term bone marrow cultures. Eur J Immunol 17:1473. 1987

19. Hynes RO: Integrins: Versatility. modulation. and signaling in cell adhesion. Cell 69:11. 1992

20. Cassiman JJ: The involvement of the cell matrix receptors. or VLA integrins. in the morphogenetic behavior of normal and malignant cells is gradually being uncovered. Cancer Genet Cytogenet 41:19. 1989

21. Ruoslahti E: Integrins. J Clin Invest 87:1. 1991

22. Humphries MJ: The molecular basis and specificity of integrin-ligand interactions. J Cell Sci 97:585. 1990

23. Shimizu Y. Van Seventer GA. Horgan KJ. Shaw S: Regulated expression and binding of three VLA (β1) integrin receptors on T cells. Nature 345:250. 1990

24. Hemler ME: VLA proteins in the integrin family: Structures, functions. and their role on leukocytes. Annu Rev Immunol 8:365. 1990

25. Long MJ: Blood cell cytoadhesion molecules. Exp Hematol 20:288. 1992

26. Winslow JM. Liesveld JL. Ryan DH. Abboud CN: Enrichment of CD34+ progenitors: Comparison of immunoadsorptive selection to flow cytometry. Blood 78:300a. 1991 (abstr. suppl 1)

27. Ryan D. Kossover S. Mitchell S. Frantz C. Hennessy L. Cohen H: Subpopulations of common acute lymphoblastic leukemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. Blood 68:417. 1986

28. Tavassoli M. Konno M. Shiota Y. Omoto E. Minguell JJ, Zanjani ED: Enhancement of the grafting efficiency of transplanted marrow cells by preincubation with interleukin-3 and granulocyte-macrophage colony-stimulating factor. Blood 77:1599. 1991

29. Wayner EA. Garcia-Pardo A. Humphries MJ. McDonald JA, Carter WG: Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J Cell Biol 109:1321. 1989

30. Hemler ME. Haung C. Takada Y. Schwarz L. Strominger J. Clabby ML: Characterization of the cell surface heterodimer VLA-4 and related peptides. J Biol Chem 262:11478. 1987

31. Elices MJ. Osborn L. Takade Y. Crouse G. Luhowskyj S, Hemler ME. Lobb R: VCAM-1 on activated endothelium interacts with the leucocyte VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 60:577. 1990

32. Smith JW. Cheresh DA: Integrin ($\alpha_v\beta_3$)-ligand interaction. J Biol Chem 265:2168. 1990

33. Saeland S. Duvert V. Caux C. Pandrau D. Favre C. Vallé A, Durand I. Charbord P. deVries J. Banchereau J: Distribution of surface-membrane molecules on bone marrow and cord blood CD34+ hematopoietic cells. Exp Hematol 20:24. 1992

34. Papayannopoulou T. Brice M: Integrin expression profiles during erythroid differentiation. Blood 79:1686. 1992

35. Simmons PJ. Mazinovsky B. Longenecker BM. Berenson R, Torok-Storb B. Gallatin WM: Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. Blood 80:396. 1992

36. Gordon MY. Clarke D. Atkinson J. Greaves MF: Hemopoietic progenitor cell binding to the stromal microenvironment *in vitro*. Exp Hematol 18:837. 1990

37. Koenigsmann M. Griffin JD. DiCarlo J. Cannistra SA: Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. Blood 79:657. 1992

38. Freedman AS. Munro JM. Rice GE. Bevilacqua MP. Murimoto C. McIntyre BW. Rhynhart K. Pober JS. Nadler LM: Adhesion

...man B cells to germinal centers *in vitro* involves VLA-4 and INCAM-110. Science 249:1030, 1990

39. Carlos TM, Schwartz BR, Kovach NL, Yee E, Rosso M, Osborn L, Chi-Rosso G, Newman B, Lobb R, Harlan JM: Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood 76:965, 1990

40. Miyake K, Medina K, Ishihara K, Kimoto M, Auerbach R, Kincade PW: A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. J Cell Biol 114:557, 1991

41. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD: An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69:927, 1992

42. Miyake K, Medina KL, Mayashi S-I, Ono S, Hamaoka T, Kincaide PW: Monoclonal antibodies to Pgp-1/CD44 block lymphohemopoiesis in long-term bone marrow cultures. J Exp Med 171:477, 1990

43. Campbell AD, Long MW, Wicha MS: Haemonectin, a bone marrow adhesion protein specific for cells of granulocyte lineage. Nature 329:744, 1987

44. Long MW, Dixit VM: Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. Blood 75:2311, 1990

45. Bernardi P, Patel VP, Lodish HF: Lymphoid precursor cells adhere to two different sites on fibronectin. J Cell Biol 105:489, 1987

46. Verfaille CM, McCarthy JB, McGlave PB: Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interactions with fibronectin. J Exp Med 174:693, 1991

47. Brunner G, Gabrilove J, Rifkin DB, Wilson EL: Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol-anchored heparan sulfate proteoglycan. J Cell Biol 114:1275, 1991

48. Sherr CJ: The colony-stimulating factor-1 receptor. Blood 75:1, 1990

49. Flanagan JG, Chan DC, Leder P: Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the SL^d mutant. Cell 64:1025, 1991

50. Kovach NL, Carlos TM, Yee E, Harlan JM: A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. J Cell Biol 116:499, 1992

51. Frelinger AL, Du X, Plow EF, Ginsberg MM: Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. J Biol Chem 266:17106, 1991

52. Upadhyaya G, Guba SC, Sih SA, Feinberg AP, Talpaz M, Kantarjian HM, Deisseroth AB, Emerson SG: Interferon-alpha restores the deficient expression of the cytoadhesion molecule lymphocyte function antigen-3 by chronic myelogenous leukemia progenitor cells. J Clin Invest 88:2131, 1991

53. Gordon MY, Atkinson J, Clarke D, Dowding CR, Goldman JM, Grimsley PG, Siczkowski M, Greaves MF: Deficiency of a phosphatidyl inositol-anchored cell adhesion molecule influences haemopoietic progenitor binding to marrow stroma in chronic myeloid leukemia. Leukemia 8:693, 1991

54. Arnaout MA, Wang EA, Clark SC, Sieff CA: Human recombinant granulocyte-macrophage colony-stimulating factor increases cell-to-cell adhesion and surface expression of adhesion-promoting surface glycoproteins on mature granulocytes. J Clin Invest 78:597, 1986

55. Elliott MJ, Vadas MA, Cleland LG, Gamble JR, Lopez AF: IL-3 and granulocyte macrophage colony-stimulating factor stimulate two distinct phases of adhesion in human monocytes. J Immunol 145:167, 1990

56. Weller PF, Rand TH, Goelz SE, Chi-Rosso G, Lobb RR: Human eosinophil adherence to vascular endothelium mediated by binding to vascular cell adhesion 1 and endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci USA 88:7430, 1991

57. Ostergaard E, Flodgaard H: A neutrophil-derived proteolytic inactive elastase homologue (hHBP) mediates reversible contraction of fibroblasts and endothelial cell monolayers and stimulates monocyte survival and thrombospondin secretion. J Leuk Biol 51:316, 1992

58. Tavassoli M, Hardy CL: Molecular basis of homing of intravenously transplanted stem cells to the marrow. Blood 76:1059, 1990